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Comparative Analyses of Mechanistic Differences Among Antiestrogens*

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ABSTRACT

Antiestrogens such as tamoxifen are one of the most effective methods of treating estrogen receptor (ERα) positive breast cancers; however, the effectiveness of this therapy is limited by the almost universal development of resistance to the drug. If antiestrogens are recognized differently by the cell as it has been suggested, then in disease conditions where tamoxifen fails to function effectively, a mechanistically different antiestrogen might yield successful results. Although many antiestrogens have been developed, a direct comparison of their mechanisms of action is lacking, thus limiting their utility. Therefore, to determine if there are mechanistic differences among available antiestrogens, we have carried out a comprehensive analysis of the molecular mechanisms of action of 4-hydroxy-tamoxifen (4OHT), idoxifene, raloxifene, GW7604, and ICI 182,780. Using a novel set of peptides that recognize different surfaces on ERα, we have found that following binding to ERα, each ligand induces a distinct ERα-ligand conformation. Furthermore, transcriptional assays indicate that each ERα-ligand complex is recognized distinctly by the transcription machinery, and consequently, antiestrogens vary in their ability to inhibit estradiol- and 4OHT-mediated activities. Relative binding assays have shown that the affinity of these ligands for ERα is not always representative of their inhibitory activity. Using this assay, we have also shown that the pharmacology of each antiestrogen is influenced differently by hormone binding proteins. Furthermore, GW7604, like ICI 182,780, but unlike the other antiestrogens evaluated, decreases the stability of the receptor. Overall, our results indicate that there are clear mechanistic distinctions among each of the antiestrogens studied. However, GW7604 and ICI 182,780 differ more significantly from tamoxifen than idoxifene and raloxifene. These data, which reveal differences among antiestrogens, should assist in the selection of compounds for the clinical regulation of ERα function. (*Endocrinology* **140:** 5828–5840, 1999)

ESTRADIOL, although principally considered a reproductive hormone, has been shown to have biological activity in bone, the cardiovascular system, and in the central nervous system (1–3). Whereas most of these biological activities were initially considered to be mediated by the ligand-dependent transcription factor ERα, the recent discovery of ERβ, a second estrogen receptor isoform, has extended our understanding of ER-biology (4–6). As yet, specific functions for ERβ in tissues other than the ovary have not been defined, suggesting that it may have a more specific role in ER-action than its ERα counterpart (7). Thus, we have limited this study to an analysis of the pharmacology of ERα.

In the absence of ligand, ERα resides in a transcriptionally inactive form within the nuclei of target cells (8). Upon binding ligand, it undergoes an activating conformational change, an event that facilitates the formation of receptor dimers (9). These ERα dimers bind specific estrogen response elements (EREs) contained within the regulatory region(s) of target genes and modulate gene transcription (10). The ultimate biological effect of this cascade of events is determined by the cellular and promoter context of the DNA bound receptor (11, 12).

Approximately 50% of all breast cancers express elevated levels of ERα (13), and prolonged exposure to estrogen(s) is a major risk factor for breast cancer (14). Therefore, antiestrogens have become the front-line therapy in the treatment and management of ERα-positive breast cancers, where it has been shown that they can inhibit the mitogenic actions of estrogens (15–17). Tamoxifen is the most effective antiestrogen used in the treatment of these breast cancers. In the metastatic setting this translates to a tumorostatic activity lasting on average 17 months, and in the adjuvant setting tamoxifen has been shown to decrease the incidence of second primary tumors by 35%. Although originally considered an antiestrogen, tamoxifen is now classified as a selective estrogen receptor modulator (SERM) because it functions in some tissues as an antagonist, while in others, like bone and the cardiovascular system, it can manifest partial or full agonist activity. The ability of tamoxifen to function as an agonist in some settings may explain why resistance to the antiestrogenic actions of this compound arises within 2 yr in the majority of patients with metastatic ERα-positive breast tumors. Specifically, it has been proposed that there may be cells within the breast tumor that are able to recognize tamoxifen as a mitogenic agent, giving these cells a selective growth advantage over their inhibited neighbors.

Interestingly, recent clinical data have revealed that tamoxifen-resistant ERα-positive cancers respond successfully

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to treatment with the ERα pure antagonist ICI 182,780 (18), but unsuccessfully when treated with the ERα partial agonist raloxifene (19). We hypothesize that cross-resistance occurs between antiestrogens having similar mechanisms of action, and that tamoxifen-resistant tumors will respond to mechanistically different antiestrogens. By inference, different antiestrogens would also find utility in the treatment of other estrogenopathies such as osteoporosis, uterine fibroids, and endometriosis as well as ERα-positive, yet tamoxifen nonresponsive, breast cancers (20). Although a large number of SERMs have been developed, a direct comparison among them to evaluate mechanistic differences has not been performed. Furthermore, even though some of the antiestrogens have been studied individually, most of the information necessary for a comparison of differences among antiestrogens is lacking due to differences in the systems, sources, and the contexts in which these studies have been carried out. Evaluation and classification of antiestrogens/SERMs into distinct groups, based on their mechanisms of action, will provide important criteria that can be used in the selection of treatment methods for clinical regulation of ERα function.

We have established a series of *in vitro* and cell-based assays to identify potential mechanistic differences among a diverse group of clinically important antiestrogens. To this end, we have analyzed, in a step-wise manner, differences exhibited by antiestrogens. This includes measurements of competitive binding to ERα, analysis of the consequent changes that occur in ERα conformation, and determination of the mechanisms by which the transcription machinery distinguishes among different ERα-ligand complexes. In addition, we have evaluated the influence of these ligands on receptor stability and the manner in which serum binding components modulate antiestrogenic activity. Our results indicate that the mechanism of action of each antiestrogen evaluated is unique; however, some antiestrogens differ more significantly from 4OHT than others.

Materials and Methods

Biochemicals

General laboratory reagents, 17β-estradiol, 4OHT, and streptavidin were purchased from Sigma (St. Louis, MO). ICI 182,780 was a gift from Dr. A. Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). Raloxifene was a gift from Dr. E. Larson (Pfizer, Inc., Groton, CT). Idoxifene was a gift from Dr. M. Gowan (SmithKline Beecham Pharmaceuticals, King of Prussia, PA). GW7604 was a gift from Dr. T. Willson (Glaxo Wellcome Inc. Research and Development, Research Triangle Park, NC). H222 (monoclonal antibody raised against human ERα) was a gift from Dr. G. Greene (Ben May Institute, University of Chicago, Chicago, IL). Secondary antibodies, Hybond-C Extra transfer membranes, x-ray film and [^{35}S]-methionine was obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). Recombinant Human ERα was purchased from PanVera Corp. (Madison, WI). Anti-M13 antibody coupled to horseradish peroxidase (HRP) was purchased from Pharmacia & Upjohn (Piscataway, NJ). The biotinylated vitellogenin estrogen response element (Biotin-GATCCCGCAGGTCACAGTGACCTG) was synthesized by Genosys Biotechnologies Inc. (Woodland, TX). Immulon 4 plates were purchased from VWR (Ocala, FL).

Cell culture and transient transfection assays

All transfections were performed using reagents and media purchased from Life Technologies, Inc. (Grand Island, NY) and sera were purchased from HyClone Laboratories, Inc. (Logan, UT). MCF-7 cells were maintained in DMEM supplemented with 8% FCS. HeLa and HepG2 cells were maintained in MEM supplemented with 8% FCS. Ishikawa cells were maintained in DMEM-Ham's F12 medium supplemented with 8% FCS and 1% sodium pyruvate, glutaMAX-1 and penicillin-streptomycin. Cells were plated in 24-well plates (coated with gelatin for transfections of HepG2 cells) 24 h before transfection. DNA was introduced into cells by transfection using Lipofectin. Briefly, triplicate transfections were performed using 3 mg of total DNA. For standard transfections, 100 ng pCMV-βGAL (normalization vector), 2000 ng reporter (either C_3-Luc, ERE_3-TATA-Luc, or ERE-TK-Luc), and 900 ng receptor (pRST7-hER (21), or ERα-AF1 (22)) or control vector pBSII-KS (Stratagene) were used for each triplicate. Cells were transfected for 3 h, at which time medium was removed and induced with the appropriate hormone diluted in phenol red-free medium supplemented with 8% charcoal-stripped FCS. Incubation with hormone continued for 24 or 48 h, after which cells were lysed and assayed for luciferase and β-actosidase activity as previously described (23). Statistical analysis of data (*t* test-paired) was done using StatView 4.5 program (Abacus Concepts, Inc., Berkeley, CA.)

MCF-7 cell proliferation assay

5,000 cells were seeded per well in 24-well plates on day 0 in estrogen-free maintenance medium. The cells were treated on days 3 through 6 with test media containing estradiol and antiestrogens at the indicated concentrations. On day 7, cells were dissolved and assayed for DNA content as described previously (24).

Relative binding affinity (RBA) assay in MCF-7 cells

RBA analyses were conducted as competition assays against 1 nM [^{3}H]estradiol in serum-free medium (SFM) and 2 nM [^{3}H]estradiol in 8% CFS-containing medium as described previously (25, 26). Briefly, MCF-7 cells were plated in 24-well plates at least three days before assay in estrogen-free medium. For each assay, cells were incubated with 0.5 ml of test media per well at 37 C and 5% CO_2 for 1 h with shaking every 15 min. At the end of the incubation, the cells were washed, resuspended in 1 ml of 10 mM EDTA (pH 12.5), neutralized with 0.1 ml of 0.77 M KH_2PO_4 (final pH, 7.2) and sonicated. Aliquots were then taken for scintillation counting and measurement of DNA. DNA was measured fluorometrically in an aliquot of the sonicate using Hoechst dye 33258 as described previously (24). Calf thymus DNA was used as the standard.

Calculations. The K_i was calculated as $K_i = IC_{50} \div [1 + (F \div K_d)]$ where IC_{50} is the concentration of unlabeled competitor required to inhibit 50% of [^{3}H]estradiol binding, F is the concentration of [^{3}H]estradiol in the medium and K_d is the apparent K_d of estradiol measured in SFM or 8% serum containing medium (26). When calculating the K_i of an antiestrogen in SFM or in 8% serum containing medium, the K_i of estradiol measured in the same media in the same assay was used instead of the K_d. The RBA was calculated by dividing the IC_{50} for unlabeled reference estradiol by the IC_{50} for each antiestrogen, and this number was expressed as a percent; estradiol = 100%.

Phage enzyme-linked immunoabsorbent assay (ELISA)

Assays were conducted as previously described (27, 28). Briefly, 96 well plates were coated with streptavidin in 0.1 M sodium bicarbonate and blocked with BSA. Biotinylated ERE (2 pmol) was added to each well. Following a 1-h incubation, the plates were washed five times with 10 mM Tris-HCl pH 8.0, 150 mM NaCl containing 0.05% Tween 20, and 2 pmol ERα was added to each well. Following a 1-h incubation, the plates were washed again and the ERα ligands were added at a final concentration of 1 μM. Following a 5-min incubation period, phage were added to the wells and incubated for 30 min at room temperature. The plates were washed as described above, and the bound phage were detected with an anti-M13 antibody coupled to horse radish peroxidase (HRP). Assays were developed with ABTS (2,2'-azinobis(3-ethylbenzthiazoline-sulfonic acid) and hydrogen peroxide for 10 min and the reactions were terminated by the addition of 1% SDS. Absorbance was measured at 405 nm in a microplate reader.

Time resolved fluorescence (TRF) assay

Assays were conducted as previously described (27, 28). Briefly, Costar high-binding 384 well plates were used to immobilize ERα as mentioned above (see ELISA assay). The ERα modulators were added at a range of concentrations. Following a 30-min incubation with the modulators, 2 pmol of a europium-labeled streptavidin (Wallac, Inc., Gaithersburg, MD)-biotinylated peptide conjugate (prepared as described below) was added and incubated for 1 h. The plates were then washed and the europium enhancement solution was added. Fluorescent readings were obtained with a POLARstar fluorimeter (BMG Lab Technologies) using a < 400 nm excitation filter and a 620 nm emission filter. The europium-labeled streptavidin-biotinylated peptide conjugate was prepared by adding 8 pmol biotinylated peptide to 2 pmol labeled streptavidin. After incubation on ice for 30 min, the remaining biotin binding sites were blocked with biotin before addition to the ERα coated plate.

Western blot analysis

Cells were induced with ligand in culture media containing 8% CFS for 48 h and nuclear extracts were prepared as described previously (29). Either 10 or 100 μg of total protein was analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose membrane and probed with monoclonal antibody H222 raised against the human ERα. Complexes were detected using ECL (Amersham Pharmacia Biotech). Densitometric quantitation of ERα levels was done by using the Image Quant software program (Molecular Dynamics, Inc., Sunnyvale, CA).

Northern blot analysis

Cells were treated with ligand for 48 h as described above and total RNA was extracted using the Ultraspec RNA Isolation System following the manufacturer's protocol (Biotecx Laboratories, Inc., Houston, TX). Twenty micrograms of total RNA were separated by electrophoresis in denaturing agarose gel (2.2 M formaldehyde and 1% agarose), and transferred to a nylon filter by capillary blotting and cross-linked by UV irradiation. The blot was hybridized in QuikHyb Hybridization Solution following the manufacturer's protocol for double-stranded probes (Stratagene, La Jolla, CA). The probe was prepared with 50 ng complementary DNA (cDNA) fragments randomly labeled with [^{32}P]dCTP and was added to the QuickHyb solution with 100 mg of salmon DNA. The following cDNA fragments were used to prepare probes: the PCR product of the N terminus of pRST7-hER plasmid (600 bp) and 36B4 cDNA fragment (708 bp) excised with *Pst*I from the pBR322 vector (provided by P. Chambon, Institut de Genetique et de Biologie Moleculaire et Cellulaire, France). The membrane was washed successively with 2 × SSC buffer and 0.1% (wt/vol) SDS solution for 15 min at room temperature and with 0.1 × SSC buffer and 0.1% (wt/vol) SDS for 30 min at 60 C. The blot was exposed to film for autoradiography and quantitated.

Results

Discordance between antiestrogen potency in competitive inhibition of estradiol-mediated activity and binding affinity to ERα

Antiestrogens effectively inhibit estradiol-stimulated mitogenesis in the breast and endometrium. This effect is largely mediated by competitive binding of antiestrogens to ERα (22); thus, the affinity with which an antiestrogen binds ERα is an important parameter in competitive binding. However, our data indicate that antiestrogen action is more complex. We compared the ability of specific antiestrogens to competitively inhibit estradiol-mediated transcription and proliferation with their affinities for ERα. Previous studies have primarily used *in vitro* systems with cellular extracts or overexpressed exogenous ERα to measure ERα affinity, and compared these data to the ability of the test compound to inhibit estrogen-mediated transcription and/or proliferation in intact cells. In this study, we have evaluated these activities in intact breast and endometrial cancer cells, which express endogenous ERα, in an effort to compare antiestrogens under conditions that are more representative of *bona fide* target cells. This parallel approach has the advantage that it equalizes the effects of the following: 1) the ability of the hormone to penetrate the cell and reach the receptor; 2) the effect of hormone binding proteins within serum on the pharmacology of the ligands; and 3) the effects of target cell metabolism on both physiological antagonism and ER binding. We have included in these mechanistic studies most of the new antiestrogens that are currently under development (Fig. 1).

As a first step in our analysis, we measured the ability of antiestrogens to competitively inhibit the transcriptional ac-

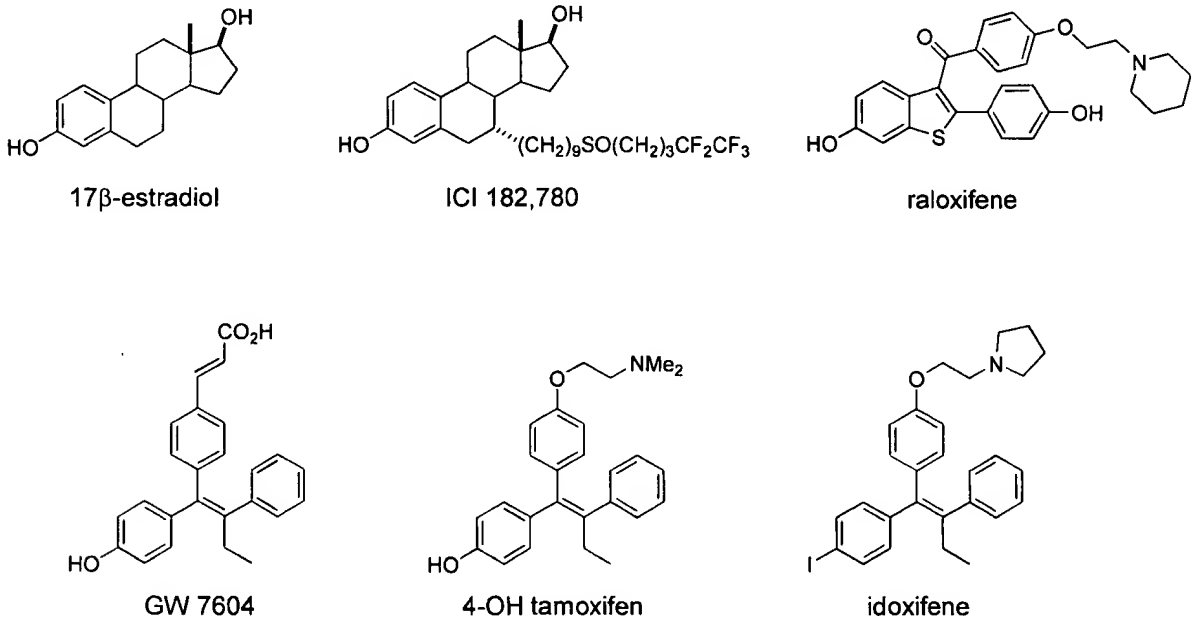

FIG. 1. Chemical structures of the ERα ligands evaluated in this study.

tivity mediated by estradiol-bound endogenous ERα in the human breast cancer cell line MCF-7 (Fig. 2A). For this purpose, a reporter construct containing three copies of vitellogenin ERE (ERE_3-TATA-Luc) was transiently transfected into MCF-7 cells and induced with 10 nM estradiol in the presence of increasing concentrations of each antiestrogen. Because endogenous levels of ERβ have not yet been detected in the cell lines that we used, this assay measures the ability of the compounds to inhibit through ERα in an endogenous setting. ICI 182,780 was the most potent inhibitor of ERα-mediated transcriptional activity (Fig. 2A), and the relative order of potencies obtained from this study was ICI

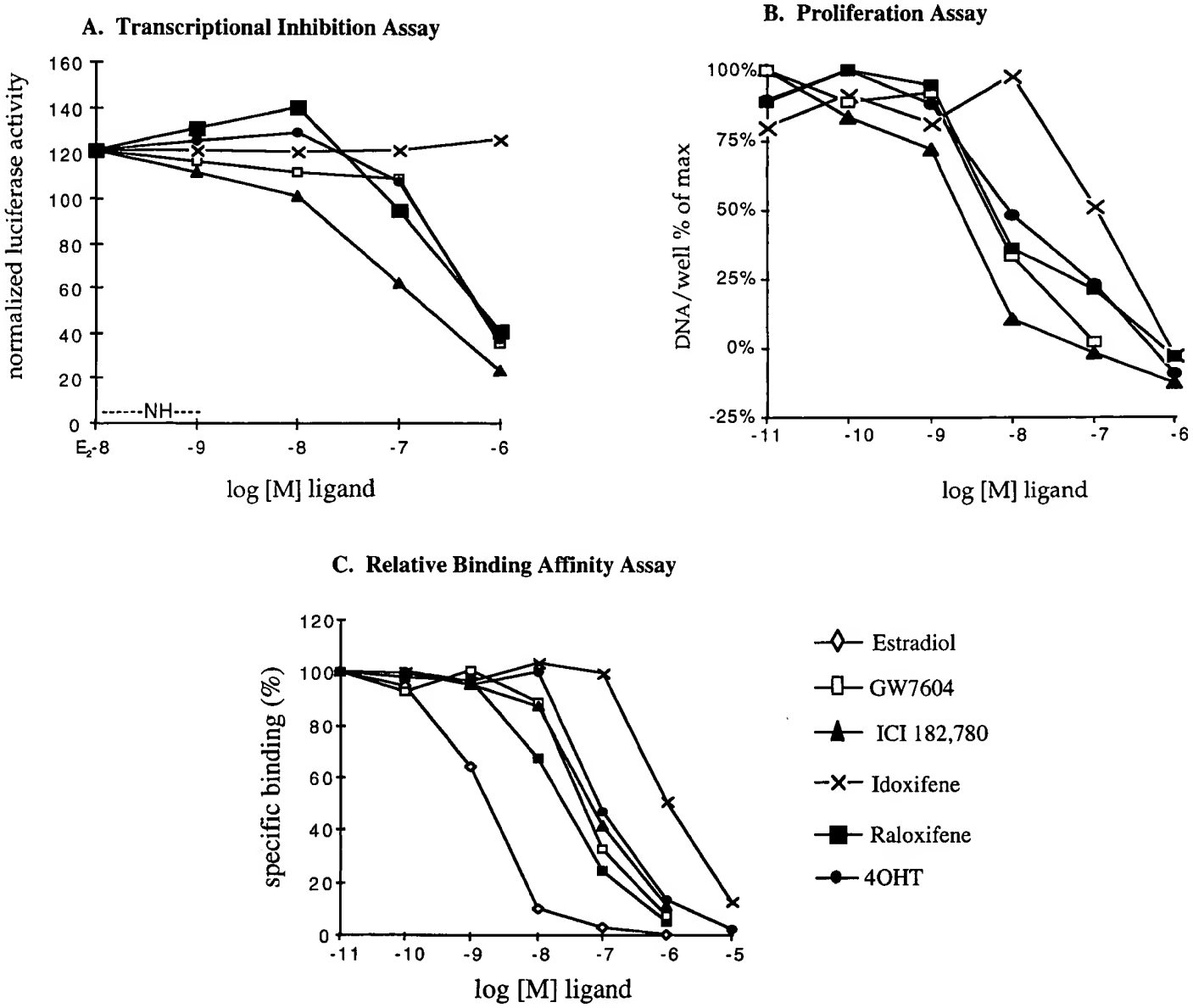

FIG. 2. Comparison of the potency and affinity for ERα of a series of antiestrogens. A, Transcriptional inhibition assay, MCF-7 cells were transiently cotransfected with 2 mg/ml of the ERE_3-TATA-Luc reporter plasmid and 0.1 mg/ml of pRSV-β-galactosidase expression vector (as an internal control for transfection efficiency). Upon transfection, cells were incubated for 24 h in the presence of 10 nM 17β-estradiol and increasing concentrations of each antagonist as indicated in culture medium. Subsequently, the transfected cells were assayed for luciferase and β-galactosidase activity. The normalized luciferase activity was calculated by dividing the absolute luciferase activity ($\times 10^4$ U) for each point by the β-galactosidase activity [(A575 × 10^5)/time in minutes]. Each data point in this experiment represents the average of triplicate determinations of the transcriptional activity under given experimental conditions for this assay. The average coefficient of variation at each hormone concentration was <10%. B, Cell proliferation assay: 5,000 MCF-7 cells were seeded per well in 24-well plates on Day 0 in estrogen-free maintenance medium. The cells were treated on Days 3 through 6 with test media containing (0.1 nM) estradiol and antiestrogens at the indicated concentrations. On Day 7, cells were dissolved and assayed for DNA content. C, RBA assay: These assays were conducted using intact MCF-7 cells in the same culture medium as in (A) Antiestrogens competed against 2 nM [^{3}H]estradiol for binding to ERα. Specific binding was determined by subtracting the nonspecific binding as measured in the presence of 200 nM estradiol. Specific binding averaged 5.97 ± 0.49 fmol/μg DNA (mean ± SE) and nonspecific binding averaged 7.7% ± 0.21% of total binding in culture medium. Each assay point was conducted in triplicate and each assay was repeated thrice.

182,780 > GW7604, raloxifene, 4OHT > idoxifene. This same order of potencies was observed in assays performed in the human endometrial adenocarcinoma Ishikawa cell line (data not shown). Furthermore, the order of potencies with which these antiestrogens inhibit estradiol mediated MCF-7 cell proliferation (Fig. 2B), in a completely endogenous system, was similar to the above order of potencies.

We next measured the relative binding affinities (RBA) of these compounds to ERα using whole cell binding assays. This study was designed to compare in parallel the relative affinities with the relative potencies of antiestrogens under the same culture conditions in intact MCF-7 cells on endogenous ERα levels. MCF-7 cells were incubated with a fixed concentration (2 nM) of [^{3}H]estradiol in the presence of increasing concentrations of each of the antiestrogens. In this RBA assay, raloxifene exhibited the highest affinity for ERα relative to estradiol (Fig. 2C, Table 1). The RBA values obtained for each ligand can be summarized as raloxifene > GW7604, 4OHT, ICI 182,780 > idoxifene. For some antiestrogens, potency differed from that expected based upon binding affinity alone. For instance, although ICI 182,780 is the most potent inhibitor of ERα-mediated transcriptional activity and cell proliferation, raloxifene shows the highest affinity for ERα (the RBA values we obtained differ from published values (30, 31) most likely due to differences in the methodology employed for this measurement). These data indicate that antagonist potency is influenced not only by receptor binding affinities, but also by processes that permit the cell to distinguish among different ERα-ligand complexes.

Another parameter that affects the pharmacology of antiestrogens is hormone-binding proteins contained within serum. Whereas estradiol and some ligands bind these proteins with high affinity (24), others do not, affecting their bioavailability, and therefore their ability to reach intracellular ERα. Hence, as an additional step, we focused on how hormone-binding proteins in serum affect the ability of ligands to reach intracellular ERα. To assess this effect, we measured the RBA in the presence (culture media) and absence of serum as described above. Our data indicate that the potency of GW7604 (4OH metabolite of GW5638) was greatly reduced in the presence of serum in the amounts present in tissue culture medium (Table 1; compare columns 2 and 3.) This may be due to the presence of the -COOH group on GW7604 and the ability of serum albumin to bind to acidic compounds (32, 33). Conversely, the RBAs of other compounds were increased in the presence of serum, suggesting that their entry into the cell from serum is greater than that of estradiol, presumably due to less binding to serum proteins. However, the magnitude of change from the presence to the absence of serum for each antiestrogen is distinct, implying different levels of binding to these serum proteins. We conclude from this experiment that binding factors contained within serum have distinct effects on the overall pharmacological profile of antiestrogens, and that comparison of potency in live cells to ERα affinity must control for these effects.

TABLE 1. Effects of hormone binding proteins on the relative binding affinity (RBA) of ligands for ERα

Ligand	RBA	
	Culture media	Serum-free media
Estradiol	100%	100%
GW7604	3.67 ± 0.27%	29.02 ± 6.30%
ICI 182,780	3.04 ± 0.27%	0.80 ± 0.25%
Idoxifene	0.26 ± 0.05%	0.11 ± 0.01%
Raloxifene	8.24 ± 1.41%	4.32 ± 0.72%
4OHT	3.66 ± 0.84%	2.75 ± 0.26%

RBA assays were conducted using MCF-7 cells where antiestrogens competed with 2 nM [^{3}H]estradiol either in culture media or serum-free media for binding to ERα. Each assay point was conducted in triplicate, and each assay was repeated thrice.

Different ligands induce distinct conformational changes in ERα

It has been demonstrated that the ability of estrogens and antiestrogens to manifest different biological activities in different cells is determined in part by the ability of individual compounds to affect ERα-conformation (9, 10). It is believed that different ERα-ligand conformations may recruit different cofactors that ultimately determine the pharmacology of each ligand. Indeed, recent crystallographic studies of ERα have demonstrated that raloxifene and 4OHT contact a different set of amino acids in the hormone binding domain than does estradiol, and evoke a unique conformational change that prevents certain cofactors from binding (34, 35). These results expand upon earlier biochemical data, obtained from partial proteolysis, indicating that ERα agonists and antagonists have different effects on ERα-structure (9, 10). Even though these crystallographic studies and partial proteolysis experiments have shown differences among agonist and antagonist bound ERα, neither study has been able to discern significant conformational differences among various antiestrogen-bound ERα complexes. We have developed a different approach to assess ERα-conformations in the presence of different ligands (28). Specifically, we have previously reported the use of phage display technology, to identify eight classes of bacteriophage that express surface peptides whose ability to interact with ERα is dependent on the conformation of a specific ER-ligand complex. Although some of the phage identified clearly interact with *bona fide* coactivator and/or corepressor binding sites on ERα, each of the peptide binding sites may represent a potential protein-protein interaction surface.

We have assessed the binding of each of the eight classes of phage-expressed peptides to ERα-ligand complexes as a probe for the effect of various ligands on ERα-conformation. This was accomplished by using an ELISA assay that measures the interaction of individual phage with each ligand-ERα complex (Fig. 3A). In this manner a specific "fingerprint" was obtained for each compound (Fig. 3B) indicating that the different SERMs evoke unique conformational changes within ERα. For instance, the phage probe α/β III interacts well with ERα in the presence of 4OHT, but interacts poorly with raloxifene- or idoxifene-ERα. The same probe shows no detectable binding to ICI 182,780- or GW7604-ERα complexes, differentiating these two compounds from the other antiestrogens. Further differentiation was accomplished using the α/β V probe where the probe bound only

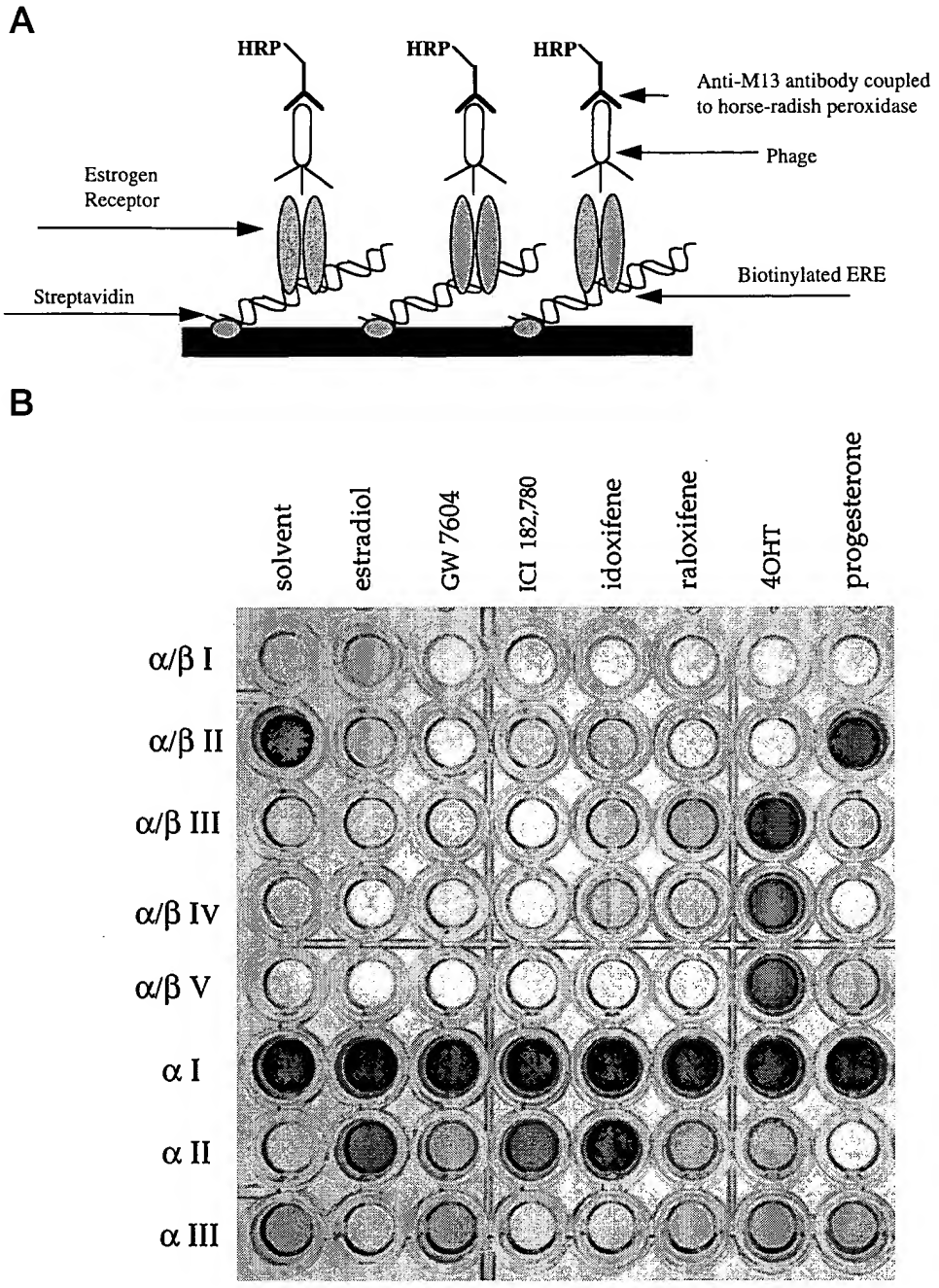

FIG. 3. Effect of ligands on ERα conformation. A, Phage ELISA assay: biotinylated vitellogenin ERE (2 pmol) was immobilized on 96-well plates coated with streptavidin. Following this step, ERα (3 pmol) was immobilized on ERE and incubated in the presence of ligand (1 μM) for 5 min before the addition of phage expressed peptides. Phage were incubated for 30 min at room temperature, and washed five times to remove unbound phage. The bound phage were detected using an anti-M13 antibody coupled to HRP and developed in ABTS and hydrogen peroxide. Assays were allowed to develop for 10 min and the reaction was terminated by the addition of 1% SDS. Absorbance was measured at 405 nM. B, The binding specificity of phage probes to ERα was evaluated as described above.

4OHT-ERα, separating the raloxifene- and idoxifene-ERα conformations from 4OHT-ERα conformation. Because 4OHT, GW7604, and idoxifene have the same triphenylethylene core structure, the unique fingerprint of each compound suggests that even minor modifications in the structure of the ligand can have a profound influence on ERα conformation. However, it was interesting that in 3/5 instances, the peptides that interacted with 4OHT-ERα, also interacted with idoxifene- and raloxifene-ERα. This observation suggests that peptide interacting surfaces on these ligand-conformations may be similar. Using the α III probe, it is possible to discriminate between the ICI 182,780- and GW7604-ERα where the probe binds to the GW7604 bound receptor but not the ICI 182,780 bound receptor. (We have also included the nonspecific binding pattern of α I probe to contrast between specific and nonspecific binding). Each ligand studied induced a distinct ERα conformation and a distinct pattern of interaction with peptides.

The different ERα-ligand conformations suggested that for each ERα-interacting probe, a different surface of interaction is exposed. Therefore, we sought to quantitate the extent to which a peptide-binding surface was exposed upon formation of the ERα-ligand complex. For this purpose, we selected the α II probe that interacts with ERα in the presence of each of the ligand used in this study. This analysis was performed using time-resolved fluorescence spectroscopy as described by Paige *et al.* (28), where the interaction between the labeled peptide and ERα was mea-

sured in the presence of increasing concentrations of ligand. The α II binding capacity of estradiol-bound ERα was set at 100. Our results (Table 2) indicate that the exposed binding surface [as represented by relative binding capacity (RBC)] of ERα for α II is different for each ligand. These results suggest that, upon binding different ligands, ERα exposes different peptide-interacting surfaces, some of which may enable the receptor to associate differentially with various coregulatory proteins.

The transcriptional machinery distinguishes among ERα-antiestrogen complexes in a ligand- and cell-specific manner

Based on the observation that each ligand induces a distinct ERα-ligand complex, it remained to be determined if, in classical ERα-responsive systems, the transcriptional machinery is capable of distinguishing among the distinct conformations. To this end, we measured the transcriptional activity of endogenous ERα in MCF-7 cells (Fig. 4A) following treatment with various ligands. This cell line was transiently transfected with ERE_3-TATA-Luc promoter and treated with solvent or a specific ligand. Under these conditions, we observed that all antiestrogens, with the exception of GW7604 and ICI 182,780, act as partial agonists exhibiting approximately 10–15% of the agonist activity of estradiol (at saturating concentrations, when compared with basal response in the absence of ligand, each compound displayed statistically significant partial agonist responses ($P < 0.05$)). A parallel analysis in Ishikawa cells gave similar results except that GW7604 acted as a partial agonist in these cells (data not shown). Although it is not possible to predict from these *in vitro* studies if the minimal level of agonist activity observed is physiologically relevant, it may partially explain why ICI 182,780 is a better inhibitor than the other antiestrogens in inhibiting estradiol-mediated activity, as described above. The observation that different ligands manifest varying levels of activity in the cell lines used here indicates that the transcription machinery does not recognize all ligand-induced ERα conformations equally.

To further dissect the transcriptional differences shown by different ERα-ligand complexes, we used a specific ERα mutant. Previous studies have indicated that receptor conformation may affect ERα-transcriptional activity by modulating cooperative interactions between the two major transcriptional activation functions within the receptor, AF1 in the amino terminus and AF2 in the carboxy terminus. These activation domains can function independently in some contexts but, for the most part, full ERα agonist activity requires both activation domains. Based on previous studies from our laboratory and others, we propose that all of the known ERα antagonists inhibit ERα-AF2 activity (22, 36, 37). Additionally, it was determined that the residual partial agonist activity manifested by antagonists like tamoxifen, is due to their ability to facilitate activation by AF1 in contexts where a functional AF2 is not required. It is likely, therefore, that the ability to regulate AF1 activity is a major discriminator among antiestrogens. Hence, evaluation of the ability of a compound to regulate ERα-transcriptional activity in contexts where AF1 alone is functional, is likely to serve as a surrogate for molecular differences among ERα-antagonists. To test this hypothesis, we have developed an assay to evaluate AF1 transcriptional activity. Specifically, we have determined that ERα-AF1, but not AF2, activity is required for ERα transcription on the complement 3 promoter (C3) when assayed in the liver hepatocellular carcinoma cell line HepG2. In this AF1-dependent environment, we assessed the response of the antagonists under evaluation following transfection with a vector expressing either ERα-wt or a mutant protein (ERα-AF1) in which AF2 has been mutated. Our results indicate that, when assayed on ERα-wt, 4OHT acts as a partial agonist exhibiting approximately 30% of the efficacy of estradiol (Fig. 4B). Under these conditions, raloxifene and ICI 182,780 function as inverse agonists, suppressing the basal transcriptional activity of the C3 promoter below the solvent control. On the other hand, GW7604 and idoxifene yield a biphasic response by exhibiting marginal partial agonist activity at 1 nM concentration ($P < 0.05$ at 1 nM compared with basal value) and by acting as inverse agonists at higher concentrations.

A parallel analysis of ERα-AF1, in which ERα-AF2 has been disrupted by three specific mutations, gave significantly different results from that obtained using wild-type ERα (Fig. 4C). Specifically, although raloxifene and ICI 182,780 behaved as inverse agonists on ERα, on ERα-AF1 raloxifene exhibited about 25% of the agonist activity of estradiol, whereas ICI 182,780 showed no agonist activity. The switch in the transcriptional activity of raloxifene probably indicates that in addition to inhibiting AF2, this compound can inhibit AF1 by a mechanism(s) that requires a functional AF2. This ERα mutant clearly differentiates raloxifene and ICI 182,780 and highlights their mechanistic differences. The ERα-AF1 mutant also separated GW7604 from idoxifene, two compounds with similar activity on ERα. Specifically, on ERα-AF1 idoxifene exhibited about 15% of the activity of estradiol, whereas GW7604 was inactive. In contrast, 4OHT was active on both ERα and ERα-AF1, as previously shown (9). We conclude that all the antiestrogens tested here use unique mechanisms of action to modulate transcription through ERα. These mechanistic differences of antiestrogens are likely to manifest distinct activities in different cells and function differently when assessed *in vivo*.

TABLE 2. Binding of the α II peptide to ERα in the presence of different ligands; an evaluation of differences in the peptide binding surfaces of ERα-ligand complexes

Ligand	Relative binding capacity
Estradiol	100
GW7604	39
ICI 182, 780	70
Idoxifene	134
Raloxifene	56
4OHT	32

ERα was immobilized on 96-well plates as described in Fig. 3A. Each ligand was added in a range of concentrations, followed by the addition of fluorescence labeled α II peptide. Relative binding capacity is defined as the maximal stimulation of signal over background achieved for a given ligand as a percentage of the stimulation by estradiol.

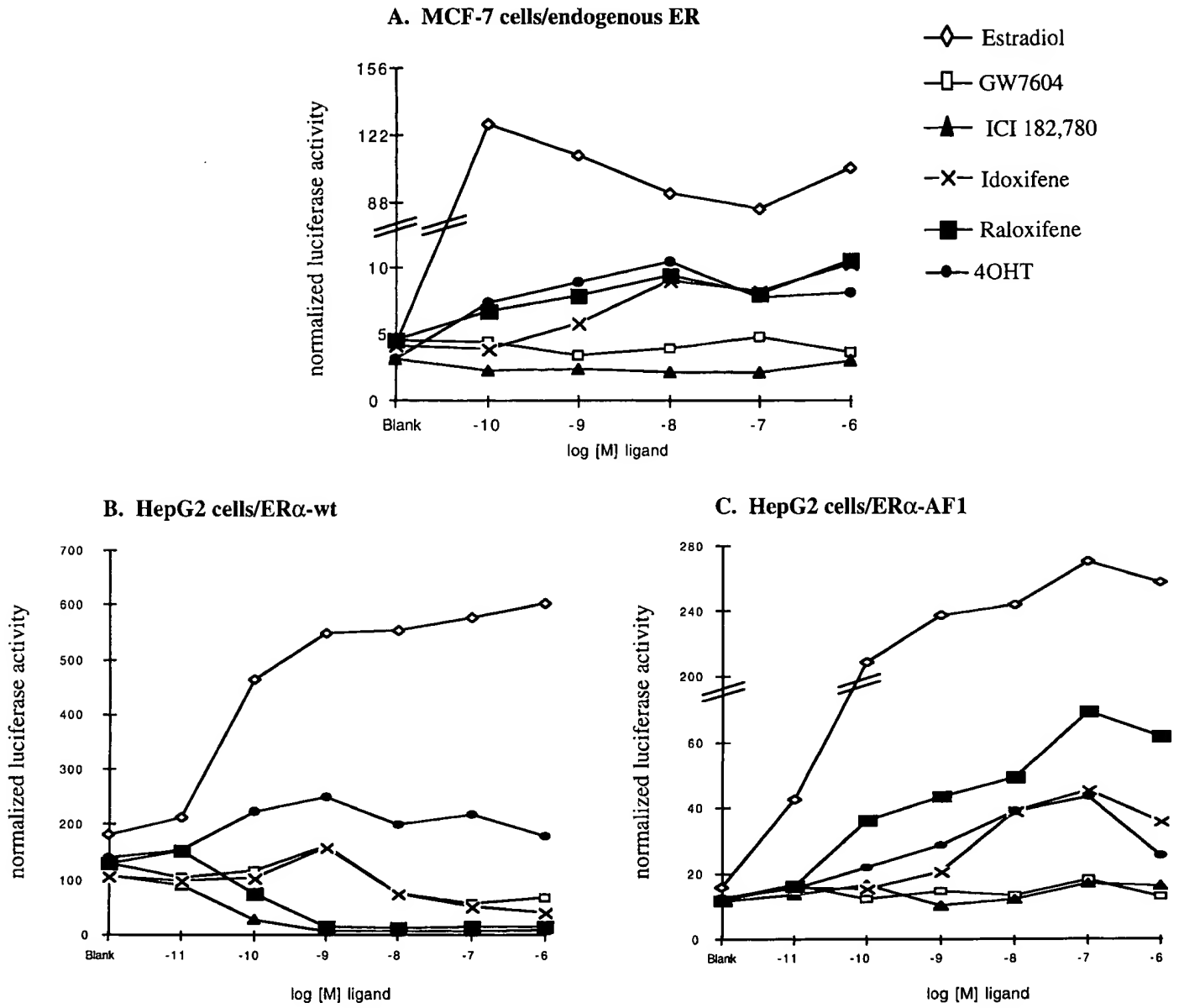

FIG. 4. The transcription machinery can distinguish among different ERα-ligand complexes. A, MCF-7 cells were transiently cotransfected with 2 mg/ml of the ERE_3-TATA-Luc reporter plasmid; 0.1 mg/ml of pRSV-β-galactosidase expression vector and control vector (pBSII-KS) to adjust the total amount of DNA to 3 mg/ml. HepG2 cells were transiently transfected with 0.9 mg/ml of a vector expressing either (B) human ERα (pRST7ER) or (C) an ERα mutant (ERα-AF1) in which the AF2 function has been inactivated (ERα-AF1) together with 2 mg/ml of the estrogen-responsive complement 3 (C3) promoter fused to luciferase gene and 0.1 mg/ml of pRSV-β-galactosidase expression vector. Upon transfection, cells were incubated for 48 h in the presence of solvent alone or increasing concentrations of estradiol or antiestrogens as indicated. Subsequently, the transfected cells were assayed for luciferase and β-galactosidase activity. Each data point in this experiment represents the average of triplicate determinations of the transcriptional activity under given experimental conditions for this assay. The average coefficient of variation at each hormone concentration was <10%.

The partial agonist activity of 4OHT can be inhibited by mechanistically distinct antiestrogens

The series of analyses we have done so far have provided compelling evidence to support the hypothesis that all antiestrogens tested in this study have unique effects on ERα-conformation and ERα-mediated transcription, and distinct efficacies as competitive inhibitors of estradiol function. Similar to previous studies (9), we also demonstrated that all antiestrogens are capable of delivering ERα-ligand complexes to DNA (data not shown). Next, we wanted to determine if the partial agonist activity displayed by tamoxifen could be competitively inhibited by the other antiestrogens tested. The rationale for this study was the observation that tamoxifen-resistant cancers display a dependence on tamoxifen for growth (30), indicating a cellular alteration that allows 4OHT to act as a partial agonist and not as an inhibitor of estradiol action. A successful cell-based, tamoxifen-resistant system had not yet been developed; therefore, we selected two systems in which 4OHT acts as a partial agonist on endogenous ERα. Specifically, MCF-7 and Ishikawa cells

were transiently transfected with ERE_3-TATA-Luc reporter and induced with a saturating concentration (10 nM) of 4OHT in the presence of increasing concentrations of each test compound. Under these conditions, in both cell types, ICI 182,780 was the most potent inhibitor of 4OHT partial agonist activity (Fig. 5, A and B), and the only antiestrogen capable of completely inhibiting this partial agonist activity. GW7604 displayed better inhibition of 4OHT partial agonist activity than idoxifene and raloxifene in both cell lines studied. Although idoxifene and raloxifene were not very successful as antitamoxifen agents in MCF-7 cells, they showed better inhibitory effects in Ishikawa cells. We conclude that ICI 182,780 and GW7604 are more efficient inhibitors of the partial agonist activity of 4OHT. This observation suggests that the partial agonist activity of tamoxifen that is thought to limit its long-term chemotherapeutic efficacy, may be circumvented by the use of mechanistically distinct antiestrogens.

Antiestrogens affect ERα expression and stability

Having established differences among antiestrogens along the individual steps in the ERα-signal transduction, we next focused on the effect of each ligand on the stability of the receptor. It has been shown previously, that ERα is targeted for proteolysis upon binding the pure antiestrogen ICI 182,780 (38). Thus, we assessed whether the stability of ERα is influenced similarly by mechanistically different antiestrogens. We examined the effects of the test compounds on endogenous ERα expressed in either MCF-7 or Ishikawa cells. The cells were incubated for 48 h in the absence or presence of compound, and then the relative nuclear ERα content was measured by immunoblotting using the H222 monoclonal antibody. The results of a typical experiment performed in MCF-7 cells (Fig. 6A) indicated that relative to solvent control, there was an increase in ERα levels in cells treated with idoxifene or 4OHT. Raloxifene did not appear to have an effect on ERα levels. However, treatment with estradiol, GW7604 or ICI 182,780 greatly reduced the receptor levels. These changes in ERα content were not due to a redistribution of the receptor within target cells, because similar results were observed when ERα content was measured in total cell extracts (data not shown). The ability of different antiestrogens to affect ERα expression was not restricted to the breast cancer MCF-7 cells as we obtained similar results when the analysis was performed in the endometrial Ishikawa cell line (data not shown). However, the extent to which estradiol, GW7604, and ICI 182,780 decreased the ERα level in Ishikawa cells was consistently less than that observed with MCF-7 cells. These results are consistent with the hypothesis that the stability and/or expression level of

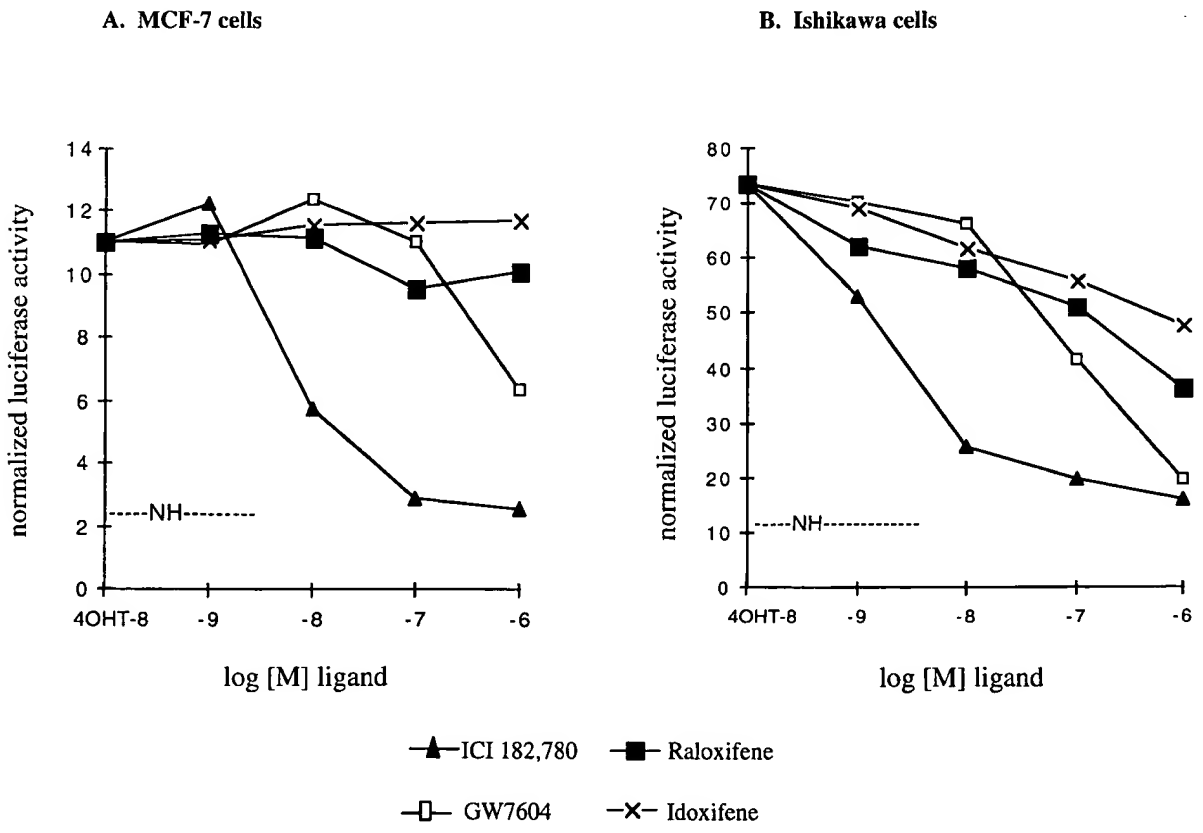

FIG. 5. Antiestrogens differ in their ability to inhibit the partial agonist activity of 4OHT. MCF-7 cells (A) or Ishikawa cells (B) were transiently cotransfected with 2 mg/ml of the ERE_3-TATA-Luc reporter plasmid, 0.1 mg/ml of pRSV-β-galactosidase expression vector and pBSII control vector to adjust the total amount of DNA to 3 mg/ml. Upon transfection, cells were incubated for 24 h in the presence 10 nM 4OHT and increasing concentrations of each antagonist as indicated. Subsequently, the transfected cells were assayed for luciferase and β-galactosidase activity. Each data point in this experiment represents the average of triplicate determinations of the transcriptional activity under given experimental conditions for this assay. The average coefficient of variation at each hormone concentration was <10%.

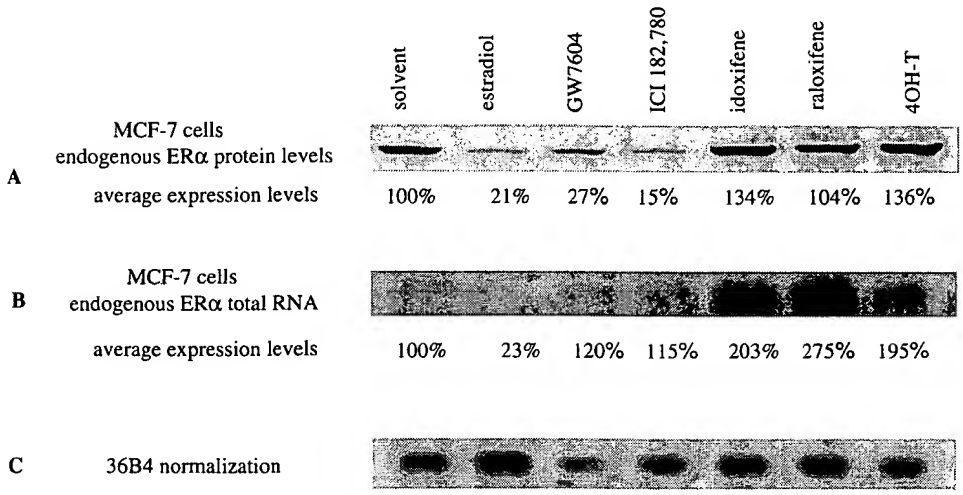

FIG. 6. Analysis of ERα protein and RNA content in target cells following treatment with agonists or antagonists. The chosen cell lines were incubated for 48 h in the presence of solvent or 10 nM estradiol or antiestrogen as indicated. Nuclear extracts were prepared, and equal amounts of protein samples were separated by denaturing PAGE, transferred to a nylon membrane, and the relative expression of ERα following these treatments was estimated by immunoblot using the ERα-specific monoclonal antibody H222. A, Averaged endogenous nuclear ERα content of MCF-7 cells (10 μg/lane). B, The cell lines were treated under the conditions mentioned above and were harvested for isolation of total RNA. Northern blots were performed as described in *Materials and Methods*. C, The 36B4 probe was used to normalize for total RNA. The ERα levels were quantitated by densitometry of immunoblots. The results shown are representative of multiple experiments performed under the same conditions.

ERα is affected by the nature of the bound ligand and the cellular milieu.

The ERα measurements performed thus far provided an assessment of the effects of ligands on endogenously expressed ERα protein. They did not, however, address the mechanisms by which the observed changes in receptor levels are achieved, *i.e.* altered receptor stability, messenger RNA (mRNA) stability, or an alteration in the rate of transcription of the ERα-gene promoter. To examine this issue, we extracted total RNA from MCF-7 cells under the treatment conditions used for Western analysis, and used Northern analysis to measure the impact of each antiestrogen under investigation on the expression of ERα-mRNA. After normalization (Fig. 6C), whereas estradiol decreased ERα mRNA levels, idoxifene, raloxifene, and 4OHT increased ERα-mRNA levels (Fig. 6B). ICI 182,780 and GW7604 did not show a significant effect on ERα-mRNA levels, implicating a posttranscriptional mechanism(s) in the regulation of ERα expression levels. We conclude from these studies that the reduced ERα expression level observed in cells treated with ICI 182,780 and GW7604 is most likely due to instability and subsequent degradation of the ligand-ERα complex. However, whether receptor down-regulation is required for the antiestrogenic action of ICI 182,780 or GW7604 remains to be determined. Cumulatively, these experiments indicate that ERα antagonists can be split into two families: those which decrease ERα protein levels (ICI 182,780 and GW7604), and those which increase or have no effect on receptor protein levels (idoxifene, 4OHT, and raloxifene).

Discussion

The classical models of ERα-action suggest that the role of ligand is that of a switch converting ERα from an inactive to an active form, implying a very simple pharmacology. It is thus considered that all agonists are quantitatively the same and, when corrected for affinity, should be indistinguishable. By inference, these models also suggest that all antagonists are the same, functioning solely by competitively blocking agonist access to the receptor. The results of our studies, however, indicate that not all antiestrogens are the same and that the cellular environment of the ERα-antagonist complex determines efficacy. These findings have important clinical implications. For instance, with reference to the development of tamoxifen-resistance in breast cancer, we propose that during the course of treatment, tumors that initially respond to tamoxifen undergo specific alterations that allow them to recognize tamoxifen as an agonist, and display a selective growth advantage over their unaltered neighboring cells (39). This may occur because either a specific enabling cofactor is overexpressed, the expression level of a corepressor decreases, or either class of protein is modified (40). Whatever the reason, the realization that different antiestrogens are not affected equally by receptor-associated proteins lends support to the hypothesis that tamoxifen-resistant tumors will respond to mechanistically different antiestrogens. To this effect, in a recent clinical trial, 69% of advanced tamoxifen resistant breast cancer patients responded favorably to treatment with ICI 182,780 (18). Accordingly, treatment of tamoxifen-resistant cancers as well as those conditions where the occurrence of adverse effects upon treatment with tamoxifen are evident (such as uterine hyperplasia), with antiestrogens that are mechanistically distinct from tamoxifen, may yield favorable results. The success of this approach relies on our ability to distinguish among available antiestrogens. Therefore, to facilitate drug evaluation and selection, we performed a head-to-head analysis of the molecular mechanisms of action of a series of clinically important SERMs/antiestrogens. The results of our studies revealed, that not all antiestrogens are the same. In addition however, they permitted an evaluation of the differences among these

TABLE 3. Summary of mechanistic differences among the antiestrogens evaluated in this study

	Solvent	Estradiol	GW7604	ICI 182,780	Idoxifene	Raloxifene	4OH-Tamoxifen
Potency in MCF-7 cells:							
Inhibition of E2-mediated transcriptional activation (IC_{50}) (M)	N/A	N/A	6.26×10^{-7}	1×10^{-7}	Did not reach IC_{50}	7.5×10^{-7}	6.36×10^{-7}
Relative binding affinity	N/A	100%	3.67%	3.04%	0.26%	8.24%	3.66%
Effect of serum on RBA relative to estradiol (RBA in SFM/RBA in 8% serum)	N/A	1.0	0.13	3.78	2.47	1.91	1.33
ERα conformation as assessed by affinity peptides							
Relative binding capacity of α II	N/A	100	39	70	134	56	32
Transcriptional activation with exogenous ERα in HepG-2 cells							
ER wt	No activity	Agonist	Inverse agonist	Inverse agonist	Inverse agonist	Inverse agonist	Partial agonist
ER-AF1	No activity	Agonist	No activity	No activity	Partial agonist	Partial agonist	Partial agonist
Transcriptional activation with endogenous ERα							
MCF-7 cells	No activity	Agonist	No activity	No activity	Partial agonist	Partial agonist	Partial agonist
Ishikawa cells	No activity	Agonist	No activity	Partial agonist	Partial agonist	Partial agonist	Partial agonist
Inhibition of 4OH-tamoxifen partial agonist activity							
MCF-7 cells	N/A	N/A	Partial inhibitor	Strong inhibitor	No inhibition	Weak inhibitor	N/A
Ishikawa cells	N/A	N/A	Strong inhibitor	Strong inhibitor	Weak inhibitor	Weak inhibitor	N/A
Ability of ERα-ligand complex to bind DNA[a]	No	Yes	Yes	Yes	Yes	Yes	Yes
Effect on nuclear ERα protein content							
MCF-7 cells	100%	21%	27%	15%	134%	104%	136%
Effect on ERα total RNA content							
MCF-7 cells	100%	23%	120%	115%	203%	275%	195%

[a] Even though data for this assay was not demonstrated in this study, the assay was conducted essentially as described previously (9).

antiestrogens, an activity that will be useful in predicting the clinical utility of specific compounds.

Previous studies have shown that it is the ability of the cell to distinguish among different receptor conformations that occur upon ligand binding that ultimately defines the tissue-specific activity of a ligand (21). To this end, in this study we have shown that: 1) ERα conformation is influenced differently by each ligand; 2) distinct peptide binding surfaces are exposed in ERα upon binding different ligands; and 3) the affinity with which peptides interact with these different ERα conformations is affected by the nature of the bound ligand. These observations suggest that different ERα-ligand complexes may interact with different coactivator/corepressor proteins within the cell. Indeed, a series of different ERα-associated coactivators and corepressors has already been identified. These coactivators, such as SRC-1 and GRIP1, potentiate the activity of ERα when coexpressed in cells (41–44). Furthermore, SRC-1 when overexpressed will convert tamoxifen from a pure antagonist to a partial agonist, whereas overexpression has no affect on the pharmacology of ICI 182,780 (45). This observation probably reflects the affinity with which SRC-1 interacts with different ERα-ligand complexes and/or the ability of the ERα-ligand conformation to recruit the coactivator. The corepressors NCoR and SMRT have been shown to associate with ERα and, although it remains to be demonstrated for ERα, previous studies have shown that PR-antagonist efficacy correlates with the relative affinity of the PR-ligand complex for either NCoR or SMRT (46, 47). Importantly, from the perspective of this study, it was shown that receptor conformation is the most important single factor regulating these interactions. These observations suggest that ERα conformation strongly influences the ability of the receptor to interact with ERα-associated cofactors. Emerging from these latest findings is

a model in which we propose that the agonist/antagonist activity of a given compound is influenced by the cellular expression level of these receptor-associated proteins. For instance, it has been shown that decreased levels of NCoR correlate with the development of tamoxifen resistance in a mouse model system for human breast cancer (40). We believe that this is a key finding that may explain the tissue-selective agonist/antagonist activity of tamoxifen, and also the reason why cells that originally recognize tamoxifen as an antagonist can switch to recognizing the compound as an agonist.

We have also shown that ERα stability within target cells is influenced by the nature of the bound ligand. We speculate that certain ERα-ligand complexes such as those formed by ICI 182,780 and GW7604 target ERα for degradation. The conformation of the ligand-receptor complex may both regulate ERα turnover and contribute to antagonist efficacy. It must be noted that we have limited this study of ERα stability to two different cell lines. It is possible that the ligand-induced receptor degradation pattern will differ in other target cells. For instance, because GW7604 has been shown to be bone protective *in vivo* (48), contrary to ICI 182,780 (49), it would be interesting to see the effect of these ligands on ERα in bone. Whatever is the case, these results suggest that ligand- ERα structure and stability are integrally linked.

Based on our overall results (as summarized in Table 3), GW7604 and ICI 182,780 appear to be two functionally unique antiestrogens whose mechanisms of action are distinct from tamoxifen. Therefore, alterations in receptor associated proteins that affect the function of ERα-4OHT may not have a significant impact on the pharmacology of GW7604 or ICI 182,780. Therefore, we believe that these compounds may be effective in the treatment of ERα positive, tamoxifen-resistant, breast cancers. Similarly, we predict that additional responders to treatment will be found among the 30% ERα positive, tamoxifen-nonresponsive breast cancers. Although functionally unique, raloxifene and idoxifene act in a manner similar to tamoxifen; therefore, the same cellular alterations that limit the efficacy of 4OHT-function could have an impact on the function of these agents. We would add as a cautionary note, however, that the separation of compounds into mechanistically different groups, based on their molecular mechanisms of action, has been assessed using the parameters we understand at the current time. Thus, as our understanding of antagonist action evolves, we may need to fine-tune and modify these classifications. Although it has proven difficult in the past to relate *in vitro* findings to clinical outcomes, it appears that cross-resistance is most likely to occur among compounds that are mechanistically similar. Thus, studies that help to separate SERMs into functionally different groups will provide science based criteria for the selection of drugs for clinical evaluation.

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